

Evaluation of a toxicogenomic approach to the local lymph node assay (LLNA)

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Genomic technologies have the potential to enhance and complement existing toxicology endpoints; however, assessment of these approaches requires a systematic evaluation including a robust experimental design with genomic endpoints anchored to traditional toxicology endpoints. The present study was conducted to assess the sensitivity of genomic responses when compared with the traditional local lymph node assay (LLNA) endpoint of lymph node cell proliferation and to evaluate the responses for their ability to provide insights into mode of action. Female BALB/c mice were treated with the sensitizer trimellitic anhydride (TMA), following the standard LLNA dosing regimen, at doses of 0.1, 1, or 10% and traditional tritiated thymidine (³HTdR) incorporation and gene expression responses were monitored in the auricular lymph nodes. Additional mice dosed with either vehicle or 10% TMA and sacrificed on day 4 or 10, were also included to examine temporal effects on gene expression. Analysis of ³HTdR incorporation revealed TMA-induced stimulation indices of 2.8, 22.9, and 61.0 relative to vehicle with an EC₃ of 0.11%. Examination of the dose-response gene expression responses identified 9, 833, and 2122 differentially expressed genes relative to vehicle for the 0.1, 1, and 10% TMA dose groups, respectively. Calculation of EC₃ values for differentially expressed genes did not identify a response that was more sensitive than the ³HTdR value, although a number of genes displayed comparable sensitivity. Examination of temporal responses revealed 1760, 1870, and 953 differentially expressed genes at the 4-, 6-, and 10-day time points respectively. Functional analysis revealed many responses displayed dose- and time-specific induction patterns within the functional categories of cellular proliferation and immune response, including numerous immunoglobulin genes which were highly induced at the day 10 time point. Overall, these experiments have systematically illustrated the potential utility of genomic endpoints to enhance the LLNA and support further exploration of this approach through examination of a more diverse array of chemicals.

Key Words: LLNA; sensitization; microarray.

Determination of the contact sensitization potential of a chemical is an important component of the safety assessment process and the murine local lymph node assay (LLNA) has emerged as the preferred assay for this evaluation (EC, 2006, 2007; ECVAM, 1999; NIH, 1999). The LLNA is an immunologically based assay that assesses the sensitization potential of a substance by monitoring the induced proliferative response of lymphocytes in the draining lymph nodes during sensitization. The observed degree of lymphocyte proliferation has been shown to correlate well with the sensitization potency of the test material (Cockshott *et al.*, 2006; Kimber *et al.*, 1994; McGarry, 2007). This assay has been extensively evaluated and validated, and possesses several advantages over existing guinea pig assays including the reduction and refinement of animal use and the generation of quantitative dose-response data which can be used to address sensitization potency (Basketter *et al.*, 2007; Haneke *et al.*, 2001; NIH, 1999; Sailstad *et al.*, 2001).

Despite these numerous advantages, and the widespread acceptance and use of the LLNA, there are areas for improvement and enhancement which are being actively researched (van Loveren *et al.*, 2008). One area has involved research into the implementation of a nonradioactive endpoint such as bromodeoxyuridine (BrdU) incorporation, lymph node cell counts, and ATP production (Ehling *et al.*, 2005; Idehara *et al.*, 2008; Suda *et al.*, 2002; Takeyoshi *et al.*, 2001). These alternative endpoints are desirable due to the fact that the use of radioisotopes requires specific facilities, handling conditions, and restrictions, which may limit assay implementation. However, these nonradioisotopic endpoints continue to be evaluated and have yet to gain full acceptance (ICCVAM, 2008a, b, c). A second area of research has investigated reducing the incidence of false positives in the LLNA which are known to occur for a limited number of irritants and certain chemical classes (Basketter *et al.*, 1998; Kreiling *et al.*, 2008). Enhanced endpoints in this area have included measuring the frequency of B220-positive lymphocytes and monitoring cell markers such as CD69, CD86, and I-A^K as well as ear swelling or inflammatory responses (Gerberick *et al.*, 1999, 2002; Homey *et al.*, 1998; Sikorski *et al.*, 1996). A third area of

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research for enhancing the LLNA has involved distinguishing between different classes of sensitizers, namely, contact (dermal) and respiratory sensitizers. In addition to identifying contact sensitizers, it has been generally accepted that respiratory sensitizers will also test positive in the LLNA, despite the fact that these compounds are rarely associated with clinical allergic contact dermatitis (Holsapple *et al.*, 2006; van Loveren *et al.*, 2008; Vanoirbeek *et al.*, 2003). Therefore, the LLNA has been considered as a potential first step for the characterization of respiratory sensitizers, followed by more specific assays to distinguish between dermal and respiratory contact sensitization potential. Specifically, a number of researches have proposed that further characterization of the T-helper cell population at the sensitization stage may be used to distinguish chemicals causing contact and respiratory sensitization. The underlying premise of this approach is the observation that contact and respiratory sensitization responses are primarily mediated by T-helper 1 (Th1) and T-helper 2 (Th2) cells, respectively (Dearman *et al.*, 2003). Endpoints explored to-date have included cytokine profiling, or fingerprinting, which involves monitoring either cytokine protein expression through enzyme-linked immunosorbent assay (ELISA) techniques or cytokine transcript levels using techniques such as quantitative real-time PCR (QRT-PCR) or ribonuclease protection assays (Dearman *et al.*, 2003). The results of initial studies have shown some promise for these approaches; however, examinations across laboratories and a wider range of compounds, with subsequent comparisons to downstream endpoints of respiratory allergy, have revealed a number of inconsistencies (Dearman and Kimber, 2002; Dearman *et al.* 2003; Plitnick *et al.*, 2002; Selgrade *et al.*, 2006; Van Och *et al.*, 2002).

A number of the approaches listed above have explored the use of specific cellular markers, cytokines, and/or gene expression responses as potential enhancements to the LLNA. Many of these endpoints were selected based on presumptions of their discriminatory potential; however, examination of a wider range of responses may identify additional endpoints with the ability to effectively enhance the LLNA. Such an approach has been facilitated by the advent of microarray technologies which offer the ability to profile the entire transcriptome in response to a chemical stimulus. The potential for toxicogenomic approaches to assess sensitization has been highlighted in a number of reviews in which the authors have noted the approach will require a systematic evaluation including a robust experimental design with genomic endpoints anchored to traditional immunotoxicology endpoints (Baken *et al.*, 2007; Luebke *et al.*, 2006). Applications of microarray approaches to assess sensitization have been conducted previously, however, the infancy of the technology resulted in incomplete transcriptome coverage, early suboptimal labeling and detection strategies, and study designs that constrained the appropriate statistical and biological interpretation of the gene expression responses (Betts *et al.*, 2003;

Hartmann *et al.*, 2006; He *et al.*, 2001). Technological advancements in biology and engineering have provided more reproducible, sensitive, comprehensive, and cost-effective microarray approaches which allow for a more accurate and complete assessment of the utility of this approach for identifying markers which may enhance the performance of the LLNA.

The objective of the current study was to further examine the use of a microarray approach to identify gene expression responses with the ability to enhance the current LLNA by providing alternative endpoints that offer increased flexibility and additional insights into mode of action. This was explored through the dose-response analysis of gene expression using the standard LLNA study design and anchoring these data to the traditional stimulation index (SI) endpoint for the assay. Temporal effects were also evaluated to further define the influence of time on the gene expression responses. The results were interpreted to assess the general utility of further exploring genomic endpoints as potential enhancements to the LLNA in terms of overall genomic responsiveness, sensitivity compared with radioisotope incorporation, and the ability to provide insights into the mechanism of action for sensitizers.

Materials and Methods

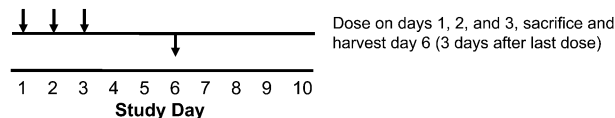
Animals. Female BALB/c mice, 8–12 weeks of age, were used in all studies (Charles River Laboratories, Inc., Kingston, NY). Mice were housed one per cage in stainless steel wire bottom cages with LabDiet Certified Rodent Diet (PMI Nutrition International, St Louis, MO) and water provided *ad libitum*. All mice were fed and handled in compliance with standards set forth by the United States Animal Welfare Act and recommendations in the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.” The procedures performed on the mice were reviewed and approved by a veterinarian and the Institutional Animal Care and Use Committee.

Experimental designs. The present examination consisted of both a dose-response and temporal analysis of gene expression in response to the frequently researched sensitizer, trimellitic anhydride (TMA; Sigma-Aldrich, St Louis, MO). The dose-response study followed the standard LLNA dosing regimen and evaluated the conventional tritiated thymidine (^3H TdR) endpoint as well as transcript responses in the draining auricular lymph nodes. Vehicle (4:1 acetone/olive oil, AOO) or one of three concentrations of TMA (0.1, 1, and 10%) were applied to the ears (25 μl /ear) of mice (nine mice per group) once daily for three consecutive days (days 1–3) followed by sacrifice on day 6. Four mice from each group were used for ^3H TdR determination of the SI, whereas the remaining five mice were used for genomic analysis. A group of untreated mice was also included to facilitate examination of vehicle-mediated responses (Fig. 1A).

For the temporal analysis, in addition to the standard day 6 time point, gene expression responses to vehicle (AOO) and 10% TMA were evaluated on study days 4 and 10. Stimulation indices were not monitored at these time points as they do not represent conventional time points for the LLNA. The day 4 group received a single dose on day 1 followed by sacrifice on day 4. The day 6 and 10 groups received three daily doses (days 1–3) followed by sacrifice on day 6 and 10, respectively (Fig. 1B). These time points were chosen to examine early and late gene expression responses relative to the standard LLNA sampling time point while facilitating comparisons to previously published reports (Betts *et al.*, 2003, 2007). For both the dose-response and time-response studies, body weight and ear erythema were evaluated prior to dosing and euthanasia.

Dose Response Study

Vehicle (A00), Untreated,
0.1, 1 or 10% TMA

**Time Course Study**

Vehicle (A00) or 10% TMA

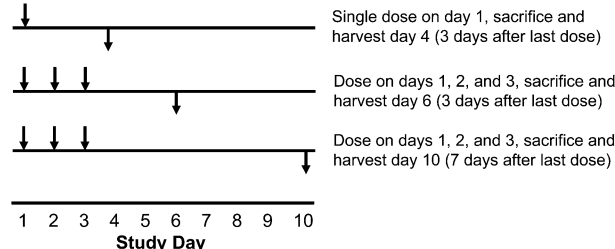


FIG. 1. Experimental design for dose-response and time-response studies. (A) The dose-response study followed the standard LLNA dosing regimen and evaluated the conventional ^3H TdR endpoint as well as transcript responses in the draining auricular lymph nodes. Vehicle (4:1 A00) or one of three concentrations of TMA (0.1, 1, or 10%) were applied to the ears of mice once daily for three consecutive days (days 1–3) followed by sacrifice on day 6. A group of untreated mice was also included to facilitate examination of vehicle-mediated responses. (B) The time-response study examined gene expression responses on study days 4, 6, and 10. The day 4 group received a single dose of 10% TMA on day 1 followed by sacrifice on day 4. The day 6 and 10 time points received three daily doses of 10% TMA (days 1–3) followed by sacrifice on days 6 and 10, respectively.

Determination of the SI. On day 6, four mice from each group in the dose-response study received an intravenous injection, via the lateral tail vein, of 250 μl of phosphate-buffered saline (PBS) containing 20 μCi of ^3H TdR (Amersham, Buckinghamshire, UK). Five hours later the mice were euthanized via carbon dioxide inhalation and the draining auricular lymph nodes for each individual mouse were excised and placed in 10 ml of PBS. Single cell suspensions of lymph node cells were prepared, washed twice with PBS, precipitated with 5% trichloroacetic acid (TCA) and refrigerated at 4°C for at least 18 h. The samples were then centrifuged, resuspended in 5% TCA and mixed with scintillation cocktail. Incorporation of ^3H -TdR was measured by β -scintillation counting and expressed as disintegrations per minute (dpm) per mouse. The SI was calculated using the mean dpm value for each dose group as the numerator and the mean dpm value from the vehicle control mice as the denominator. A test material that, at one or more concentrations, causes a threefold or greater increase in proliferation is considered to be positive in the LLNA. A measure of the sensitizing potency of TMA was determined by calculation of an EC_3 value (the estimated concentration required to induce a threshold positive response). The calculation of the EC_3 values was carried out by linear interpolation according to the equation:

$$\text{EC}_3 = c + [(3 - d)/(b - d)] \times (a - c)$$

where the data points lying immediately above and below the SI value of 3 on the LLNA dose-response plot have the co-ordinates (a,b) and (c,d), respectively (Basketter *et al.* 1999b).

RNA extractions. For examination of gene expression responses, auricular lymph nodes from individual mice were excised and placed in RNAlater as per manufacturer's instructions (Applied Biosystems, Foster City, CA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and included tissue homogenization using a TissueLyser (Qiagen) and an on-column DNA digestion with DNase I (Qiagen). The RNA was quantified using the ND-1000

spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with a Bioanalyzer RNA integrity number greater than 7 were used for microarray analyses.

Microarray gene expression analysis. Total RNA from individual mice was amplified and labeled according to manufacturer's instructions using the One-Color Low RNA Input Linear Amplification Kit Plus (Agilent Technologies). Labeling reaction input for all samples was 500 ng of total RNA. This approach uses a T7 RNA polymerase which simultaneously amplifies the target material and incorporates cyanine 3-labeled CTP (Cy3-CTP). Samples were purified and quantified to obtain the yield of cRNA and specific activity of the Cy3 incorporation. Samples with an amplified yield of less than 1.65 μg or a specific activity of less than 9.0 pmol Cy3 per μg cRNA were not hybridized to an array. For samples meeting these criteria, 1.65 μg of labeled cRNA was fragmented and suspended in hybridization buffer and hybridized onto one array of an Agilent 4 $\times$ 44K Whole Mouse Genome Oligo microarray slide. Samples were placed in a rotisserie hybridization oven at 65°C for 17 h and were subsequently washed in Agilent Gene Expression Wash Buffers. Slides were then scanned on an Agilent G2565AA Microarray Scanner using the extended dynamic range scanning mode at a 5- μm resolution. Data were extracted from the images using Agilent Technologies' Feature Extraction software version 9.5.3.

Microarray data normalization, filtering, and statistical analyses. Gene expression data were analyzed using GeneSpring GX 7.3.1 (Agilent). All data preprocessing and normalization were performed according to manufacturer and literature recommendations for optimal performance of the Agilent one-color array platform (Agilent-Technologies, 2007; Guo *et al.*, 2006; Patterson *et al.*, 2006; Shippy *et al.*, 2006). Normalization of the data was performed in GeneSpring as follows: values below 5.0 were set to 5.0, a per chip normalization to the 50th percentile value, and a per gene normalization to the median value in all samples. Prior to statistical analysis, data were prefiltered on flags (present or absent) and expression levels in order to focus the downstream analysis on the most reproducibly detectable measurements. Statistical analyses employed a parametric ANOVA approach on the dose and/or time variables followed by a Tukey's *post hoc* test to identify differential gene expression responses relative to vehicle. A false discovery rate of 0.001 was applied to both the dose-response and time-response studies after applying a Benjamini-Hochberg multiple testing correction. All data clustering and functional annotation was also performed in GeneSpring.

QRT-PCR analysis. Select microarray gene expression responses were verified using QRT-PCR. Total RNA (500 ng) was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems). cDNA was used as template in subsequent QRT-PCR assays which were conducted using predesigned TaqMan Gene Expression assay reagents on an ABI 7500 real-time PCR sequence detection system (Applied Biosystems). Relative expression between treated and vehicle control samples were determined using the comparative Ct method ($\Delta\Delta\text{C}_\text{T}$ Method) with beta actin used as the endogenous control. A full listing of all predesigned TaqMan assays used in these analyses can be found in Supplementary Table 1. Statistical analysis was performed on the ΔC_T values for each animal as these values represent the normalized dependent variables from the experimental analysis. The data were tested for equality of variance using Bartlett's test. Dose-response data were subjected to a one-way ANOVA with the factor of dose followed by Dunnett's test. Time-response data were subjected to a two-way ANOVA followed by Tukey's *post hoc* test.

Results**LLNA Responses**

Treatment with TMA did not result in dose- or time-related alterations in body weight or body weight gain. Ear erythema

was monitored as an indication of chemical irritancy before each treatment and prior to sacrifice. TMA did not result in erythema in the 0.1% dose group, whereas minimal erythema was observed on day 6 in three of nine mice in the 1% dose group. Treatment with 10% TMA resulted in minimal erythema after a single dose (day 4 group), whereas groups which received three daily doses (day 6 and day 10 groups) progressed to moderate erythema which persisted through day 6. All mice sacrificed on day 10 exhibited recovery as the erythema severity decreased to minimal at the time of sacrifice.

Cellular proliferation in the auricular lymph nodes was evaluated through $^3\text{HTdR}$ incorporation in the dose-response study on day 6 as per the standard LLNA. SI values were calculated relative to the vehicle control and yielded values of 0.9, 2.8, 22.9, and 61.0 for the untreated, 0.1, 1, and 10% TMA dose groups, respectively (Fig. 2). The $\text{EC}_{0.11\%}$ was calculated to be 0.11% which equates to a strong sensitization potential based on the European Centre for Ecotoxicology and Toxicology potency classification (Kimber *et al.*, 2003). Stimulation indices were not monitored for the day 4 and 10 dose groups as these time points do not represent conventional time points for the LLNA.

Microarray Data Filtering and Clustering

Prefiltering of the microarray data sets, based on flags (present or absent) and expression levels followed by statistical analysis, was conducted in order to focus the downstream analysis and interpretation on the most reproducibly detectable and treatment-altered responses. A full list of the significant gene expression responses for both the dose- and time-response experiments can be found in Supplementary Tables 2 and 3, respectively. An additional ± 1.5 fold change relative to vehicle was also applied to further focus the generated gene lists. This combination of statistical and fold-change filters is consistent with previously evaluated approaches for identifying reproducible gene expression responses for biological interpretation (Guo *et al.*, 2006).

Application of these criteria to the gene expression data from the dose-response study identified 9, 833, and 2122 genes which were differentially expressed between the vehicle control and the 0.1, 1, and 10% TMA dose groups, respectively. In addition, there were no differentially expressed genes identified between the vehicle control and untreated groups. The dynamic range of the gene expression responses extended from a 15-fold repression through a 52-fold induction. Examination of the common responsive genes across each of the dose groups revealed remarkable overlap indicating the consistency of the response with increasing dose, within a time point (Fig. 3A). Hierarchical clustering of the differential gene expression responses was performed to provide a high-level overview of the similarity of the gene expression responses for each treatment/dose group. This analysis revealed the expected groupings which included the

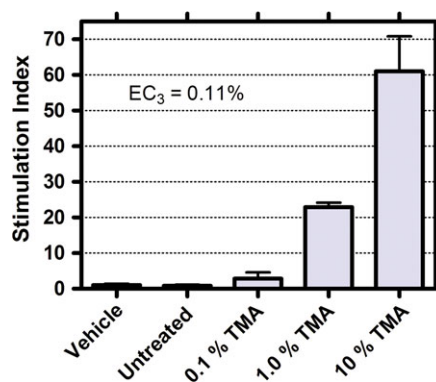


FIG. 2. SI values for each treatment group as determined by $^3\text{HTdR}$ incorporation in the auricular lymph nodes on day 6 as per the standard LLNA. Values are expressed relative to the vehicle control and represent the mean $\pm$ standard deviation. The EC_3 value, the concentration required to induce a stimulate index of 3, is also presented.

vehicle and untreated groups clustering together, along with the low dose TMA group, whereas the 1 and 10% TMA groups formed a separate cluster (Fig. 3B).

Application of the same data filtering criteria to the time-response study identified 1760, 1870, and 953 genes which were differentially expressed between the 10% TMA groups and their time-matched vehicle control groups at the 4, 6, and 10 day time points, respectively. The dynamic range of these gene expression responses extended from a 15-fold repression up to a 71-fold induction. Examination of the common responsive genes across each of the time points revealed a prominent overlap between the time points, however a number of time-specific responses were also apparent indicating the dynamic nature of the gene expression responses over time (Fig. 3C). Hierarchical clustering of these responses revealed the vehicle and TMA groups clustered separately with the day 4 and 6 TMA groups clustering more closely when compared with day 10. In addition, the day 10 TMA treated group exhibited a unique cluster of very highly induced genes (Fig. 3D).

Sensitivity of Gene Expression responses relative to Stimulation Indices

The sensitivity of the gene expression responses were compared with results obtained via $^3\text{HTdR}$ incorporation by applying the standard EC_3 approach to both endpoints. For this analysis, calculations for the gene expression data focused on significant responses at the 0.1 and 1.0% dose levels only. Examination of the data revealed 82 gene expression responses met the requirements for calculation of an EC_3 value (fold change ≥ 3). Of these responses, no genes yielded an EC_3 value more sensitive than that obtained for $^3\text{HTdR}$ incorporation (0.11%; Table 1). However, a number of responses were within this range with two genes yielding EC_3 values less than 0.2% and 23 with values less than 0.5% (Table 2). These data indicate comparable sensitivity between the $^3\text{HTdR}$ and the gene

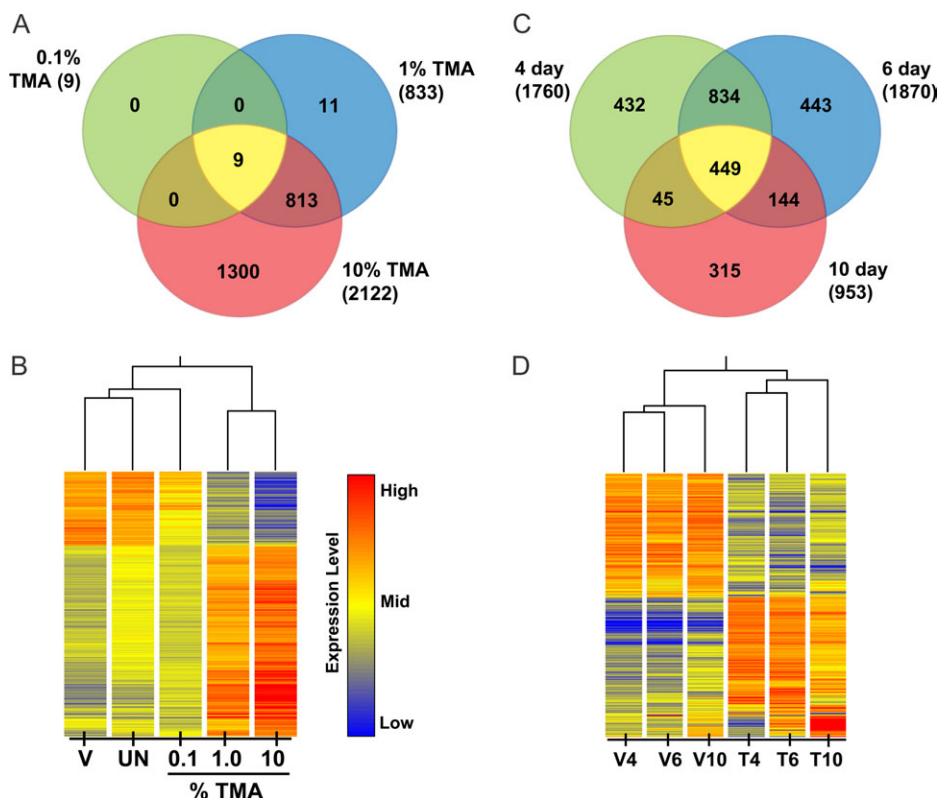


FIG. 3. Overlap of significant gene expression responses and hierarchical clustering within the dose-response and time-response experiments. (A) Venn diagram illustrating the high degree of overlap for the significant gene expression responses across doses which highlight the reproducibility of the responses. (B) Hierarchical clustering by dose group reveals the expected pattern of clustering which involved the vehicle, untreated and low dose groups clustering together and the mid and high dose TMA groups forming a separate cluster (V = vehicle treated group, UN = Untreated group). (C) Venn diagram illustrating the overlap between the different groups for the time-response study. The lower degree of overlap highlights the temporal differences in gene expression responses. (D) Hierarchical clustering by treatment and time point illustrates that the vehicle and TMA groups form separate clusters. Furthermore, the day 4 and 6 TMA responses are more similar when compared with the day 10 TMA group which displayed a small grouping of highly induced genes. Colors for hierarchical clustering figures represent the expression level intensity based on microarray analysis (V4, V6, and V10 indicate vehicle treated groups at 4, 6, and 10 days, respectively, whereas T4, T6, and T10 indicate TMA treated groups at 4, 6, and 10 days, respectively).

expression responses when using this analysis approach. It is important to note that threshold responses will vary depending on the endpoint that is being monitored and a threefold threshold may not be appropriate for gene expression responses, or other alternative LLNA endpoints, due to the lower dynamic range. For example, threshold values of 1.5 and 1.3 have been proposed for the potential alternative LLNA endpoints of cell count index and BrdU-ELISA, respectively (Ehling *et al.*, 2005; ICCVAM, 2008b). Therefore, to address this in a preliminary manner, analyses were also conducted using a 2-fold and 1.5-fold threshold for the gene expression responses. Using these values, 410 and 833 of the significant responses met the requirements for the calculation of an EC_2 and $EC_{1.5}$, respectively. Of these, 14 and 141 were below the 0.11% EC_3 observed with ^3H TdR incorporation, although they were within a similar range. It should be noted that the actual application of an appropriate threshold for a gene expression response will require assessment on a gene-by-gene approach, will vary depending on the gene of interest, and should only be determined after a rigorous analysis

of its predictive accuracy for a wide range of sensitizers and nonsensitizers.

Functional Evaluation of Gene Expression Responses

Gene Ontology was used to provide information on the attributes of the significant gene expression responses to facilitate examination of altered functional categories. The approach identified significantly altered ontologies by their over- or under-representation in the list of differentially expressed genes versus the normal incidence of the category on the array and was applied to both the dose-response and time-response studies. The most highly represented categories consisted of functional responses related to cellular proliferation, consistent with the proliferating environment of the draining lymph node after exposure to a sensitizer (Table 3). Genes in this category included the induction of various cyclins, cyclin-dependent kinases, and other genes involved in the regulation and maintenance of cell division including *Cdca5*, *Chek1*, *Kntc1*, and *Mki67*. Coordinately, a number of

TABLE 1
Comparison of the Sensitivity of Gene Expression Responses to the ³HTdR Incorporation Endpoint^a

EC _x ^b	No. of qualifying genes ^c	Range of EC _x values ^d	Mean EC _x	No. of values below the ³ HTdR EC ₃
3	82	0.12–1.00	0.71	0
2	410	0.03–1.00	0.60	14
1.5	833	NA ^e –1.00	0.40	141

^aTo focus on only the most sensitive gene expression responses, only the 0.1 and 1% TMA dose groups were examined in this evaluation.

^bEC_x is defined as the estimated concentration to result in an “x-fold” increase in lymph node proliferation.

^cQualifying genes are those where the induction or repression exceed the designated threshold determined by the EC_x value.

^dWhere appropriate, the log linear equation was applied.

^eNot available as some values approached zero due to limitations of the extrapolation approach at this threshold level.

genes associated with anti-proliferative activity were down-regulated including Bmp2 and 7, Cables1, and Cdkn1c.

As expected after exposure to a chemical sensitizer, additional overrepresented functional categories included genes related to the development and regulation of immune response and T-cell activation (Table 3). This included the induction and repression of various cytokines and cytokine receptors as well

as other genes which are known to be associated with a developing immune and allergic response including Ccr4, Gata3, Irf4, and Zbtb32. Interestingly, a more focused analysis on the highly upregulated group of genes at the 10 day time point revealed the top overrepresented functional category was humoral immune response (Table 4). This category included various immunoglobulin genes such as Igh-4 (IgG1), Ighg, Igh, Igk, and Igj as well as other genes that play a key role in development of an antigen-specific immune response such as Ada, Aicda, Slpi, and Timd2. The upregulation of these genes highlights the continued development of a specific immune response to TMA at this later time point. Overall, the identified functional categories were consistent with the phenotypic alterations which would be expected in a proliferating lymph node after exposure to a sensitizer such as TMA.

QRTPCR Verification of Results

QRTPCR was used to verify changes in transcript levels for a selected subset of differentially expressed genes. Genes evaluated by QRTPCR were chosen based on functional association with sensitization and/or proliferation while also considering facilitating comparisons to previous publications. In total, 10 gene expression responses were verified by QRTPCR (Supplementary Table 1), all of which displayed expression patterns and dynamic ranges consistent with the microarray data. The dose-response data highlight the similarity in the sensitivity of the two detection strategies at low

TABLE 2
Listing of Genes with EC₃ Values Less Than 0.5% and Their Associated Fold-Change Expression Relative to Vehicle Controls

Common name	Gene symbol	Entrez gene ID	0.1% TMA	1% TMA	10% TMA	EC ₃
Interleukin 4	Il4	16189	2.87	10.00	8.74	0.12
Aldo-keto reductase family 1, member C18	Akr1c18	105349	2.10	17.33	12.26	0.15
Lectin, galactose binding, soluble 7	Lgals7	16858	1.60	14.12	14.60	0.20
Granzyme B	Gzmb	14939	1.30	10.53	31.47	0.27
Fatty acid binding protein 7, brain	Fabp7	12140	1.57	7.73	16.73	0.31
Tenascin N	Tnn	329278	1.55	7.33	12.11	0.33
Selectin, platelet	Selp	20344	1.73	6.41	6.19	0.34
RIKEN cDNA 2810055G20 gene	2810055G20Rik	77994	0.48	0.19	0.14	0.37
Interleukin 21	Il21	60505	1.14	6.96	18.76	0.39
Kinase noncatalytic C-lobe domain containing 1	Kndc1	76484	0.46	0.22	0.24	0.41
Spermatogenesis associated glutamate (E)-rich protein 6, pseudogene 1	Speer6-ps1	73266	0.90	6.81	51.39	0.42
RIKEN cDNA C330003B14 gene	C330003B14Rik	105594	1.71	5.11	9.28	0.44
Transmembrane protein 54	Tmem54	66260	0.48	0.22	0.16	0.45
Brevican	Bcan	12032	0.67	0.19	0.08	0.45
Thrombospondin 4	Thbs4	21828	1.27	5.64	7.80	0.46
Collagen and calcium binding EGF domains 1	Ccbe1	320924	0.39	0.28	0.17	0.46
FXD domain-containing ion transport regulator 2	Fxyd2	11936	0.44	0.25	0.22	0.47
Coagulation factor X	F10	14058	1.03	5.77	15.69	0.47
Mast cell protease 8	Mcpt8	17231	1.00	5.67	2.50	0.49
FXD domain-containing ion transport regulator 4	Fxyd4	108017	0.99	5.68	7.11	0.49
RIKEN cDNA 4932425I24 gene	4932425I24Rik	320214	1.62	4.82	14.98	0.49
Neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	18212	0.41	0.27	0.18	0.49
Pre T-cell antigen receptor alpha	Ptcr	19208	0.85	5.76	9.44	0.49

TABLE 3
Functional Categorization of Select Dose- and Time-Dependent Changes in Gene Expression

Functional category	Common name	Gene symbols	Entrez gene ID	Dose response ^a			Time course ^a		
				0.1% TMA	1% TMA	10% TMA	4 day	6 day	10 day
Cell cycle/cell division/DNA replication	Cyclin A2	Ccna2	12428	1.39	2.81	5.00	7.42	5.00	3.27
	Cyclin B1	Ccnb1	268697	1.51	3.39	6.51	9.74	6.51	3.40
	Cyclin B2	Ccnb2	12442	1.28	2.73	4.39	6.67	4.39	2.70
	Cyclin E1	Ccne1	12447	1.25	2.57	4.44	7.00	4.44	2.11
	Cyclin E2	Ccne2	12448	1.24	2.68	4.63	7.61	4.63	3.52
	Cell division cycle 25 homolog C	Cdc25c	12532	1.58	3.76	7.18	8.90	7.18	3.72
	Cell division cycle 6 homolog	Cdc6	23834	1.35	2.73	5.20	7.38	5.20	3.29
	Cell division cycle associated 2	Cdca2	108912	1.36	2.86	4.81	4.95	4.81	3.74
	Cell division cycle associated 3	Cdca3	14793	1.35	2.82	4.59	6.03	4.59	3.28
	Cell division cycle associated 5	Cdca5	67849	1.27	2.64	5.06	6.96	5.06	4.06
	Cyclin-dependent kinase 2	Cdk2	12566	1.18	1.64	2.11	2.44	2.11	1.34
	Cyclin-dependent kinase 6	Cdk6	12571	1.22	1.63	3.04	2.23	3.04	1.61
	Cyclin-dependent kinase inhibitor 3	Cdkn3	72391	1.33	2.74	4.68	6.07	4.68	3.07
	Checkpoint kinase 1 homolog (S. pombe)	Chek1	12649	1.28	2.42	4.46	4.89	4.46	2.76
	Helicase, lymphoid specific	Hells	15201	1.27	2.08	3.51	3.82	3.51	2.25
	Kinetochore associated 1	Kntc1	208628	1.40	2.52	4.57	5.36	4.57	3.19
	Antigen identified by antibody Ki 67	Mki67	17345	1.35	2.50	4.54	5.26	4.54	3.19
	Myeloblastosis oncogene-like 2	Mybl2	17865	1.34	2.78	5.79	6.69	5.79	3.60
	Bone morphogenetic protein 2	Bmp2	12156	0.98	0.63	0.27	0.40	0.27	0.35
	Bone morphogenetic protein 7	Bmp7	12162	0.81	0.47	0.29	0.55	0.29	0.37
	Betacellulin	Btc	12223	0.61	0.23	0.13	0.19	0.13	0.26
	Cdk5 and Abl enzyme substrate 1	Cables1	63955	0.60	0.48	0.43	0.49	0.43	0.58
	Cyclin-dependent kinase inhibitor 1C (P57)	Cdkn1c	12577	0.75	0.46	0.32	0.31	0.32	0.46
	Fibroblast growth factor 7	Fgf7	14178	0.70	0.50	0.34	0.44	0.34	0.43
	Insulin-like growth factor 2	Igf2	16002	0.63	0.34	0.18	0.25	0.18	0.37
Immune response	Caspase 3	Casp3	12367	1.22	1.88	3.26	3.12	3.26	1.49
	Chemokine (C-C motif) ligand 6	Ccl6	20305	0.65	1.90	3.14	2.52	3.14	1.31
	Chemokine (C-C motif) receptor 4	Ccr4	12773	1.19	2.78	3.50	5.49	3.50	1.64
	Complement component factor i	Cfi	12630	1.43	3.78	4.01	4.25	4.01	1.14
	Cytotoxic T-lymphocyte-associated protein 4	Ctla4	12477	1.16	1.92	3.09	3.29	3.09	1.27
	Exonuclease 1	Exo1	26909	1.26	2.34	4.28	5.59	4.28	2.71
	Fc receptor, IgG, low affinity IIb	Fcgr2b	14130	0.98	1.72	2.29	1.52	2.29	1.17
	GATA binding protein 3	Gata3	14462	1.20	1.66	2.07	2.33	2.07	0.87
	Granzyme A	Gzma	14938	1.11	2.43	5.48	4.27	5.48	1.26
	Granzyme B	Gzmb	14939	1.30	10.53	31.47	23.80	31.47	3.90
	Interferon regulatory factor 4	Irf4	16364	1.29	2.12	2.92	2.52	2.92	1.75
	Interleukin 1 receptor antagonist	Il1rn	16181	0.92	2.02	2.90	2.24	2.90	1.10
	Interleukin 21	Il21	60505	1.14	6.96	18.76	3.26	18.76	7.56
	Interleukin 2 receptor, alpha chain	Il2ra	16184	1.61	2.75	5.79	6.47	5.79	1.08
	Interleukin 4	Il4	16189	2.87	10.00	8.74	8.79	8.74	7.43
	Lymphocyte-activation gene 3	Lag3	16768	1.06	2.59	3.96	3.24	3.96	1.87
	Selectin, platelet	Selp	20344	1.34	3.65	5.20	5.51	5.20	1.40
	Zinc finger and BTB domain containing 32	Zbtb32	58206	1.42	2.25	4.82	9.36	4.82	1.44
	Autoimmune regulator	Aire	11634	1.08	0.55	0.37	0.35	0.37	0.42
	CD209b antigen	Cd209b	69165	0.81	0.56	0.25	0.42	0.25	0.25
	Glutamyl aminopeptidase	Enpep	13809	0.70	0.32	0.17	0.38	0.17	0.24
	Interleukin 11 receptor, alpha chain 1	Il11ra1	16157	0.80	0.61	0.45	0.64	0.45	0.57
	Interleukin 17D	Il17d	239114	0.56	0.42	0.29	0.41	0.29	0.57
	Orosomucoid 3	Orm3	18407	0.83	0.33	0.15	0.24	0.15	0.37

^aValues represent fold-change relative to the appropriate time-matched vehicle control.

TABLE 4
Listing of Key Immunological Response Genes which were Highly Upregulated on Day 10

Common	Gene symbol	Entrez gene ID	4 day ^a	6 day	10 day
Adenosine deaminase	Ada	11486	1.09	1.26	2.52
Activation-induced cytidine deaminase	Aicda	11628	0.37	3.23	11.13
Ig kappa	AY498738	243425	0.68	2.99	7.01
ELL associated factor 2	Eaf2	106389	0.59	1.78	10.78
IgH; IGHV1-84	EG434609	434609	0.55	1.49	8.88
IGKV4-57-1	Gm1418	384514	0.71	1.27	7.51
Immunoglobulin heavy chain complex	Igh	111507	0.76	2.65	8.84
Immunoglobulin heavy chain 4 (serum IgG1)	Igh-4	16017	0.44	3.60	70.54
Immunoglobulin heavy chain 6 (heavy chain of IgM)	Igh-6	16019	0.68	1.96	6.58
Immunoglobulin heavy chain (gamma polypeptide)	Ighg	380794	0.24	2.75	31.36
Immunoglobulin joining chain	Igj	16069	0.45	0.88	4.65
Immunoglobulin kappa chain variable 19 (V19)-14	Igk-V19-14	667881	0.50	1.14	6.09
Immunoglobulin kappa chain variable 4-63	Igkv4-63	628571	0.58	1.30	6.73
Immunoglobulin lambda chain, variable 1	Igl-V1	16142	0.94	1.08	5.63
Indoleamine-pyrrole 2,3 dioxygenase	Indo	15930	0.44	1.44	3.33
Secretory leukocyte peptidase inhibitor	Slpi	20568	0.80	2.05	11.30
T-cell immunoglobulin and mucin domain containing 2	Timd2	171284	0.46	2.45	5.43

^aValues for all time points represent fold-change relative to the appropriate time-matched vehicle control.

doses. The data for Ifng verified the lack of responsiveness of this classic Th1 gene in response to TMA, a prototypical Th2 inducing chemical (Fig. 4). The time course data highlight the gene-specific temporal responses, with Il4 and Mki67 exhibiting a sustained induction across all time points, whereas Il21 and Gzmb show peak induction at the 6-day time point (Fig. 5). In contrast, both Aicda and Igh-4 exhibited a progressive induction over time. These data provide verification of the microarray gene expression responses using an independent measurement approach and highlight the reproducibility of the measurements across these technologies.

Discussion

The present study was conducted to examine the potential utility of genomic endpoints as enhancements to the LLNA for informing the hazard assessment of potential contact sensitizers. This was accomplished through a systematic evaluation of the overall genomic responsiveness of the draining auricular lymph node, the sensitivity of the responses in comparison to the traditional radioisotope endpoint, and the ability of the data to provide potential insights into mechanisms of action.

Before exploring the use of a microarray approach to enhance a toxicological endpoint, one must demonstrate high responsiveness and reproducibility in the gene expression responses from the tissue of interest. Early studies examining toxicogenomic approaches to assess sensitization experienced difficulties with transcript detection, low dynamic range of the responses, and poor verification of array responses, which may have been related to the state of the technology and study designs that constrained the appropriate statistical and bi-

ological interpretation of the data. This is evident in the low concordance between microarray responses and other gene-specific measurement techniques, such as quantitative PCR, in early publications (Betts *et al.*, 2003; He *et al.*, 2001). The high concordance between microarray and QRT-PCR responses in the present study is likely due to technological improvements in each of the assays as well as a more complete annotation of the mouse genome. These improvements are also likely contributors to the detection of a larger number of significantly altered responses. Collectively, these improvements have provided a comprehensive pool of reproducible putative markers which may serve as potential alternative and/or enhancing endpoints for the LLNA. However, it is also important to note that there were a number of common gene expression responses identified in this study when compared with previously conducted microarray analyses of the draining lymph node. For example, Betts *et al.* (Betts *et al.*, 2003) have previously reported the induction of onzin (Plac8) and the repression of GlyCAM-1 (Glycam1) in response to the sensitizer 2,4-dinitrofluorobenzene, both of which were similarly regulated in the present study. Interestingly, Betts *et al.* also reported the induction of guanylate binding protein 2, an interferon-gamma inducible gene, which was not detected in the present study, consistent with the lack of Ifng induction. Ku *et al.* (2008) also reported a number of sensitizer-specific gene expression responses in CBA mice, including Il12rb1, Gzma, and Tfdp1, which were similarly regulated in the present study. These examples indicate the reproducibility of gene expression responses in independent studies. The consistency of gene expression responses across different microarray platforms, mouse strains, and laboratories will continue to be an important consideration as stakeholders consider the application of these

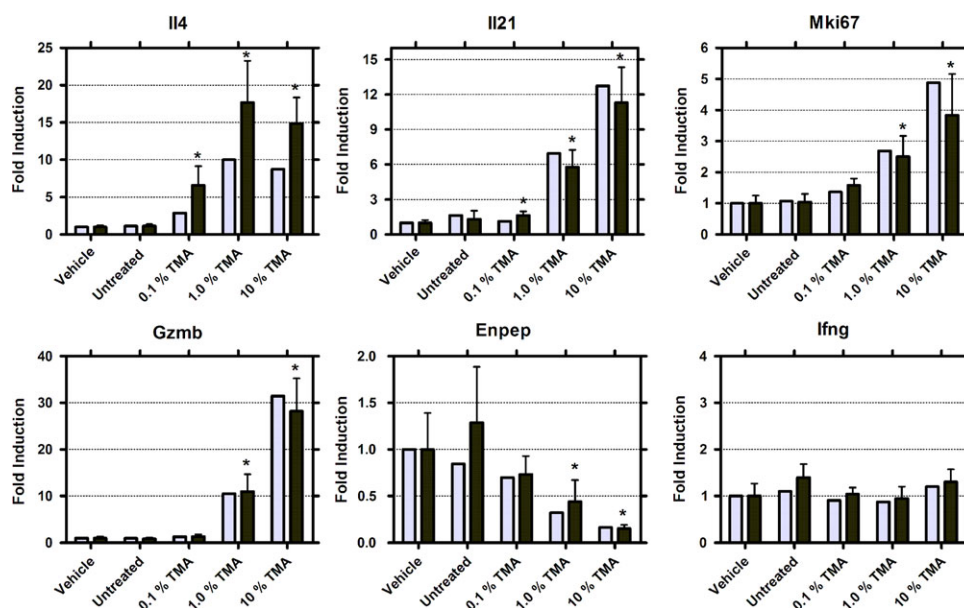


FIG. 4. QRT-PCR verification of select gene expression responses from the dose-response study. Grey bars represent the microarray data, whereas the black bars represent the QRT-PCR data. The y-axis represents the fold change calculated relative to the vehicle control group, whereas the x-axis represents the different dose groups. Data points represent the fold change $\pm$ standard deviation of at least 4 independent samples. Gene identities are denoted by their official gene symbols. *Indicates $p < 0.05$ for the QRT-PCR data.

endpoints as potential biomarkers of effect in toxicological evaluations.

Published reviews have indicated that toxicogenomics may represent a valuable tool to enhance our understanding and assessment of chemical sensitizers, while also indicating that the data from these studies should be anchored to the existing toxicological endpoints (Baken *et al.*, 2007; Luebke *et al.*, 2006). For this reason, we conducted a dose-response analysis of gene expression along-side the traditional $^3\text{HTdR}$ incorporation response in order to facilitate the comparison of these endpoints and establish their relative sensitivity. Many alternative endpoints for the LLNA have been reported to be somewhat less sensitive than the results obtained with $^3\text{HTdR}$ incorporation, which may hinder their ultimate acceptance (ICCVAM, 2008b, c). Alternatively, early predictions concerning the use of genomic endpoints indicated the expectation that they would be orders of magnitude more sensitive than traditional toxicological endpoints. The results from this study illustrate that when using a standard EC_3 calculation to compare the sensitivity, none of the genes yielded an EC_3 value more sensitive than that obtained for $^3\text{HTdR}$ incorporation, although there were a number of responses with similar sensitivity. It should also be noted that the threefold threshold for a positive response when examining $^3\text{HTdR}$ incorporation was derived via a comprehensive analysis of a wide range of sensitizers (Basketter *et al.*, 1999a) and that such a threshold may not be appropriate for a gene expression response. Furthermore, a threshold would be expected to vary on a gene-by-gene basis as the calculated value for the EC_3 in the LLNA

is not only a function of the overall sensitivity of the response but also the dynamic range (Ehling *et al.*, 2005). For example, threshold values of 1.5 and 1.3 have been proposed for the putative alternative LLNA endpoints of cell count index and BrdU-ELISA, respectively (Ehling *et al.*, 2005; ICCVAM, 2008b). Regardless, the data from this study indicate that there are a number of gene expression responses with comparable sensitivity to the $^3\text{HTdR}$ incorporation endpoint. In addition, the fact that the genomic responses are not more sensitive than the traditional toxicological endpoint is consistent with recent publications that have made similar anchored comparisons for other toxicological endpoints (Andersen *et al.*, 2008), although this effect is likely to be a tissue- and endpoint-dependent response.

The present study also examined the variable of time on the gene expression responses. Although there were differences in the significant responses between the day 4 and day 6 time points, a large proportion of the responses across these time points were common. This was also reflected in the functional analysis which revealed few differences in the functional categories identified between these two time points. By comparison, there was a greater diversity between the responses at the 6-day and the 10-day time points. This was notably displayed for a number of highly upregulated genes at the 10-day time point which were associated with the functional ontology of humoral immune response. These responses illustrate the continued development of a specific immune response to TMA at this later time point. Furthermore, these potentially mechanistically insightful responses indicate that it may be useful to

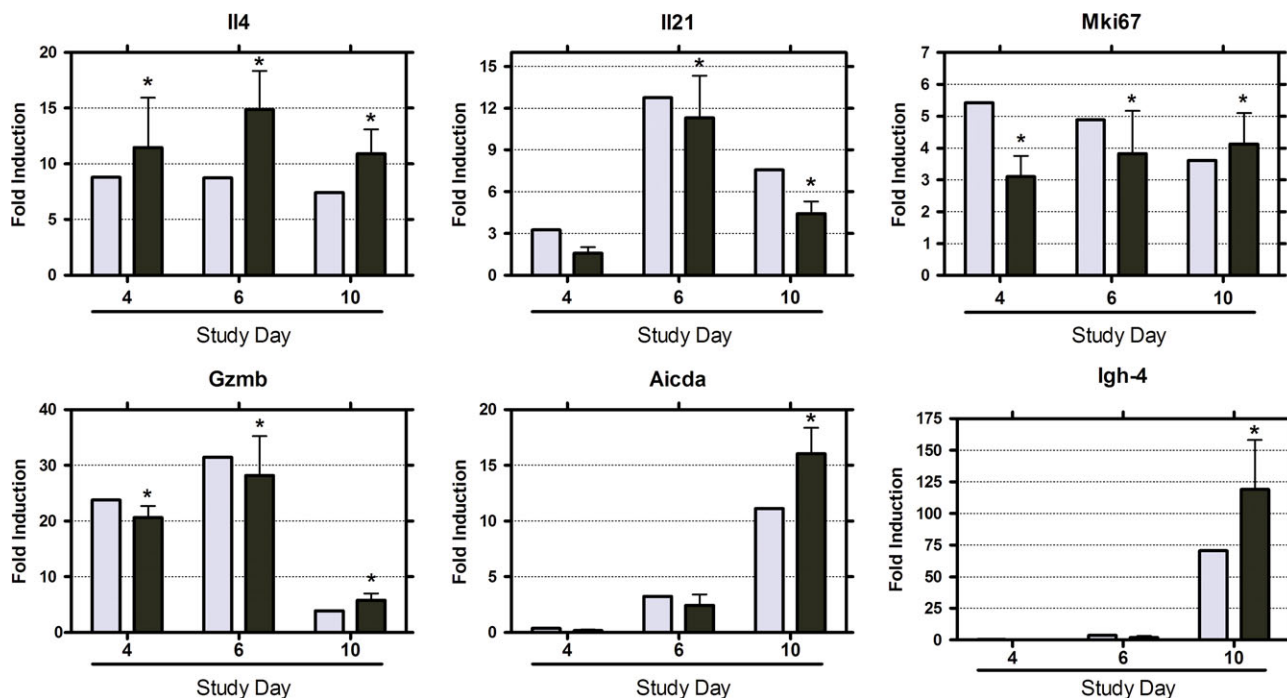


FIG. 5. QRT-PCR verification of select gene expression responses from the time-response study. Grey bars represent the microarray data, whereas the black bars represent the QRT-PCR data. The y-axis represents the fold change calculated relative to the time-matched vehicle control, whereas the x-axis represents the different time points. Data points represent the fold change $\pm$ standard deviation of at least 4 independent samples. Gene identities are denoted by their official gene symbols. *Indicates $p < 0.05$ for the QRT-PCR data.

examine both the day 6 and day 10 time points as additional chemicals are evaluated using this toxicogenomic approach.

The ultimate goal of research into toxicogenomic signatures is to provide data that will facilitate the accurate classification of materials with respect to their hazard potential and risk. Although knowledge of the functional relevance is not a requirement for the use of a gene expression response in chemical categorization, the ability to anchor the responses to known physiological phenotypes and modes of action will increase confidence in their relevance and aid in their ultimate acceptance as predictive endpoints.

Functional interpretation of the data from this study revealed that many of the responses were associated with aspects of cellular proliferation such as cell division, cell cycle regulation, and DNA replication. Regulation of this class of genes is not unexpected due to the fact that gene expression profiling was performed on the proliferating lymph node. Differentially expressed genes in this category included various cyclins, cyclin-dependent kinases, and their regulators, which collectively are involved in the control of cellular progression through different stages of the cell cycle (Sanchez and Dynlacht, 2005). Regulation of these genes is consistent with previous toxicogenomic evaluations of proliferating tissues such as the estrogen-induced uterus (Fertuck *et al.*, 2003). These responses are unlikely to provide any distinction between true-sensitizers and false-positive irritants, or for distinguishing contact and respiratory sensitizers. However,

they may have utility as indicators of the proliferative status of the lymph node which may have applications to research aimed at developing a nonradioisotopic endpoint for the LLNA.

Another major functional category represented by the differentially expressed genes in this study involved those related to immune response including, lymphocyte proliferation, T-cell activation, and humoral immune response. Genes in this category included a number of cytokines, cytokine receptors, immunoglobulins, and various other genes involved in regulating a developing immune response. These genes have the potential to serve as valuable biomarkers of effect and may have applications in distinguishing sensitizers from irritants and classifying chemicals as contact or respiratory sensitizers. The chemical sensitizer evaluated in the present study was TMA, a prototypical Th2 inducing chemical which has been associated with the development of respiratory sensitization and allergy (Sailstad *et al.*, 2003). Consistent with this, many of the gene expression responses associated with the immune response ontology were linked to the promotion of a Th2 cellular response. For example, IL4 was highly induced throughout the time course study and was the most sensitive response to TMA exposure in the dose-response study. IL4 has been well-characterized to be associated with the differentiation of T-cells to the Th2 lineage and the differentiation of B cells to produce IgE and IgG1 (O'Garra and Arai, 2000). Interestingly, the early induction of IL4 was followed by the 3- and 11-fold induction of Aicda on day 6 and 10, respectively. Aicda is an

inducible gene that plays an important role in class-switch recombination and somatic hypermutation in B cells and is induced by IL4 (Dedeoglu *et al.*, 2004; Kim *et al.*, 2007) and may mediate the subsequent class switching and differentiation of B cells to IgE and IgG1 producing cells. Another gene that may be involved in this process is Irf4 which was induced throughout the study. This gene encodes a transcription factor that is required for plasma cell differentiation as well as the induction of Aicda (Klein *et al.*, 2006; Sciammas *et al.*, 2006). Exo1 was also upregulated throughout the study and, similar to Aicda, this gene has been shown to play a key role in class-switch recombination and somatic hypermutation (Bardwell *et al.*, 2004). Importantly, these changes in gene expression were followed by the induction of various immunoglobulins on day 10 including Igh-4 (IgG1), Ighg, Igj, and a number of variable chain transcripts (Table 4). The late induction of these immunoglobulin-encoding transcripts are likely the result of the earlier induction of genes such as IL4, Irf4, Exo1, and Aicda and highlights the complex transcriptional response which occurs in the draining lymph node during sensitization.

A number of additional genes related to the development of Th2 cellular responses were also upregulated including Ccr4, Gata3, Il21, and Timd2. Ccr4 is a chemokine receptor that is preferentially induced in Th2 cells during differentiation (Morimoto *et al.*, 2005), whereas Gata3 is a Th2-specific transcription factor whose expression is essential for T-cell development and differentiation of naïve T cells into Th2 effector cells (Pai *et al.*, 2004). Il21 is a cytokine preferentially expressed in Th2 cells and has been reported to inhibit interferon-gamma production in developing Th1 cells, thereby supporting development of a Th2 response (Wurster *et al.*, 2002). Finally, Timd2 is involved in regulating T-cell proliferation and is preferentially upregulated during Th2 differentiation and is downregulated in differentiated Th1 cells (Meyers *et al.*, 2005). Importantly, in addition to the induction of various Th2 related transcripts, Th1 related transcripts such as Ifng remained unchanged, suggesting specificity of the response. Although these examples highlight the potential value of gene expression responses for elucidating putative modes of action, a complete assessment of their utility for distinguishing between sensitizers and irritants, and classifying chemicals as contact or respiratory sensitizers will only be realized after a more comprehensive analysis of a series of chemicals in these classes.

In summary, the present study has further examined the use of a microarray approach to identify enhanced endpoints for the LLNA. This was explored through the dose-response analysis of gene expression using the standard LLNA study design while also examining temporal effects to further define the influence of time on the gene expression responses. The data have indicated that the auricular lymph node displays a robust genomic response upon exposure to a chemical sensitizer and yields a number of responses of comparable sensitivity to the traditional ³HTdR endpoint. Functional interpretation of the

data indicate the association with the expected functional categories including those of cellular proliferation and immune response, with a number of genes related to the development of a Th2 cellular response. Overall, these experiments have systematically assessed the potential utility of genomic endpoints as enhancements to the LLNA. The data support further exploration of this technology through examination of a more diverse array of chemicals including irritants and contact and respiratory sensitizers.

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